

INTERACTIONS BETWEEN *Euphorbia esula* TOXINS AND BOVINE RUMINAL MICROBES

SCOTT L. KRONBERG,^{1,*} FATHI T. HALAWEISH,² MINDY B. HUBERT,³
and PAUL J. WEIMER⁴

¹*Northern Great Plains Research Laboratory, USDA-Agricultural Research Service,
P.O. Box 459, Mandan, ND 58554, USA*

²*Department of Chemistry and Biochemistry, South Dakota State University, Brookings,
SD 57007, USA*

³*West River Agricultural Center, South Dakota State University, Rapid City, SD 57702, USA*

⁴*US Dairy Forage Research Center, USDA-Agricultural Research Service, and
Department of Bacteriology, University of Wisconsin, Madison, WI 53706, USA*

(Received January 19, 2004; revised June 13, 2005; accepted September 21, 2005)

Abstract—Cattle generally avoid grazing leafy spurge (LS; *Euphorbia esula*), whereas sheep and goats will often eat it. Understanding metabolism of toxic phytochemicals in LS by bovine rumen microflora may help explain why cattle often develop aversions to LS after initially eating it. Toxicity of LS compounds after *in vitro* fermentation with normal vs. antibiotic-modified bovine rumen digesta was evaluated at different lengths of fermentation. Levels of toxic and aversion-inducing ingenols were determined for fermented and nonfermented mixtures of LS and bovine rumen digesta, and the toxicity of an aversion-inducing extract of LS to rumen microbial species that are common in cattle, sheep, and goats was evaluated. Fermentation of LS with bovine digesta increased the toxicity of extracted compounds. Introduction of neomycin (an antibiotic that preferentially inhibits gram-negative bacteria) into the LS and bovine rumen digesta mixtures did not appear to affect toxicities regardless of fermentation length. Levels of ingenol were observed in LS and bovine digesta mixtures (both fermented and nonfermented) that were consistent with levels of ingenols reported for LS. Finally, a toxic extract of LS had little or no negative effect on the growth of several common species of rumen bacteria. The results indicate that LS is not generally toxic to the ruminal bacteria, but that microbial activity in the rumen may be responsible for enhancing LS toxicity to cattle.

Key Words—Cattle, ingenol, leafy spurge, ruminal fermentation, toxicity.

* To whom correspondence should be addressed. E-mail: Kronberg@mandan.ars.usda.gov

INTRODUCTION

Leafy spurge (LS; *Euphorbia esula* L.) is a problematic invasive plant species that was introduced in North America in the 19th century and now occurs in many areas of the western United States and Canada. Grazing of LS by sheep and goats is considered to be an important management tool, but many observations indicate that cattle eat little LS (Lym and Kirby, 1987; Hein and Miller, 1992). Cattle may initially graze the plant, but they appear to develop an aversion (Kronberg et al., 1993a). In contrast, sheep and goats can maintain good productivity with considerable daily intake of LS (Landgraf et al., 1984; Walker et al., 1994; Kirby et al., 1997).

One possible reason for differences in foraging behavior among ruminant species is differences in metabolism of phytochemicals by the host's ruminal microflora (Kronberg, 1999). Ruminal microbes have the capacity to detoxify some phytochemicals and increase toxicity of others (James et al., 1975; Carlson and Breeze, 1984; Pass et al., 1984; Craig et al., 1992; Duncan and Milne, 1992). Among ruminants, there are striking differences in tolerance of plant toxicants (Smith, 1992). If diet selection differences among ruminants are a result, in part, of differential rumen metabolism of phytochemicals (Kronberg, 1999), cattle may be reluctant to graze LS because their ruminal microbes do not metabolize toxic and aversive LS phytochemicals as do ruminal microbes in goats (Kronberg and Walker, 1993). Among potential toxicants in LS, ingenol and some of its esters occur in LS and are known to induce feed aversions in cattle (Halaweh et al., 2002). Moreover, antibacterial agents are known to modify ruminal fermentation (Russell and Strobel, 1988; Wachenheim et al., 1992) and could affect the ability of the ruminal microflora to metabolize compounds in LS to a less or more toxic form. This study had three objectives: (1) to determine if antibiotic addition to bovine rumen digesta would alter the ruminal microflora such that the toxicity of LS to ruminants was decreased or increased at different lengths of fermentation; (2) to quantify ingenol and its esters in fermented mixtures of LS and bovine ruminal digesta; and (3) to determine if an aversion-inducing extract of LS inhibited the growth of several common species of ruminal bacteria, thus indicating that LS could be inducing feeding aversions simply by inhibiting forage digestion.

METHODS AND MATERIALS

Leafy Spurge Collection and Preparation. Leafy spurge was collected in its flowering growth stage in July 2000 near Veblen, SD, USA (ca. 46°N, 97°W). Stems were cut within 4 cm of the ground, and this herbage was air dried and ground in a Wiley mill through a 1-mm screen. Air-dried alfalfa

(*Medicago sativa*) was also ground in a Wiley mill through a 1-mm screen. Air-dried LS was used rather than freeze-dried because the authors have found air-dried LS to be highly aversive to cattle in several feed aversion trials. Alfalfa was used as the control plant material because it is a forb like LS, but in contrast to LS, it is readily consumed by cattle.

Digesta Collection and Preparation of Fermentation Samples. For comparison of the effects of fermentation with normal or modified bovine digesta, ruminal digesta were collected from two ruminally cannulated yearling cattle fed with a diet of alfalfa and grass (mixed cool-season grass species) hay for at least 14 d prior to digesta collection. Whole digesta from the two individuals were combined to avoid the consequences of obtaining atypical digesta from one animal, and 75 ml of mixed digesta were added to 300 ml of buffer solution (Tilley and Terry, 1963) and 25 g of ground LS or alfalfa (control) in a 500-ml Erlenmeyer flask. The flasks were agitated, purged with CO₂, plugged with vented stoppers, and incubated at 39°C with periodic agitation. For the modified cattle digesta treatment, 0.5 mg of neomycin sulfate (an antibiotic that preferentially inhibits growth of gram-negative bacteria) were added to the buffer solution immediately before it was mixed with digesta and plant material to alter the population of microbes during fermentation. Because most conditioned food aversions develop from negative feedback received within 12 hr after ingestion of food (Kronberg et al., 1993b), metabolism of leafy spurge toxins was evaluated at 0, 6, and 12 hr. To stop the fermentation, samples were frozen quickly in thin layers (ca. 1 cm) in plastic bags.

Extraction of Fermentation Samples. Fermentation samples (400 ml each) were removed from the freezer 1–2 d prior to extraction. Samples were thawed at 4°C and extracted five times in a 2-l separatory funnel with 500 ml of petroleum ether (PE; manual shaking for 8 min each). PE extracts were filtered through Whatman 50 filter paper to remove any particles and were then concentrated by using a rotary evaporator at 45°C. Concentrated samples were pipetted into vials and refrigerated for use in the Brine Shrimp Lethality Tests.

Brine Shrimp Lethality Test. Potential toxicity of extracts to ruminants was estimated by using the Brine Shrimp Lethality Test, a general bioassay for toxic plant constituents (Meyer et al., 1982; Anderson et al., 1991). PE extracts of fermentations were completely dried under N₂ gas and were then redissolved in PE to a concentration of 1 mg ml⁻¹. Cytotoxic effects of the extract samples were evaluated at 50 µg of sample per milliliter of seawater. To obtain this concentration, 250 µl of sample were pipetted into each vial, and four vials (replicates) per sample were made. Samples were dried overnight in a hood. The next day, 20 µl of dimethyl sulfoxide (DMSO) were added to each vial to dissolve the samples. Two control vials were also used that contained only 20 µl of DMSO. Up to 50 µl of DMSO may be added per 5 ml of brine before DMSO toxicity affects the results (McLaughlin et al., 1991).

Seawater was prepared in an 18.9-l tank containing 152 g of sea salt (purchased locally) dissolved in 4 l of deionized water at room temperature (22–24°C). Shrimp eggs were added to one side of the tank (separated by a perforated dividing dam), and the tank was placed near a lamp to attract the shrimp. Shrimp eggs were allowed to hatch for approximately 48 hr after setup; hatchlings migrated toward the light and through the perforated dam, where they were collected.

Fermentation extract samples were vortexed, and seawater was added to a volume of 3 ml. Ten shrimp were added to each vial, and the final volume was brought to 5 ml. Vials were placed near a lamp for 48 hr, at which time the number of live shrimp in each vial were counted.

Two toxicity assays were conducted as described above. Assay 1 evaluated shrimp toxic response to LS or alfalfa fermented with bovine rumen digesta containing the antibiotic. Assay 2 evaluated the shrimp toxic response to LS fermented with normal bovine rumen digesta or the digesta containing the antibiotic.

Statistical Analysis. The general linear models procedure of SAS (1988) was used for statistical analysis. For each assay, the model statement included treatment, time of fermentation, and the interaction term as independent variables and the number of live shrimp in each vial as the dependent variable; vial was the experimental unit. Fischer's least significant difference procedure was used to separate treatment means. The threshold *P* value for significance was 0.05.

Isolation of Ingenol. LS was collected, dried, and ground as described above and was then extracted with methanol/water (9:1). The methanol extract was evaporated under reduced pressure to yield a concentrated aqueous sample, which was extracted with PE. The PE extract was removed, and the aqueous layer was freeze-dried and hydrolyzed with 0.5 M methanolic KOH for 2 hr at room temperature to hydrolyze esters, releasing ingenol and other alcohols. The hydrolyzed sample was neutralized to pH 7.0 with 1 M HCl. After neutralization, the sample was extracted with CH₂Cl₂ to yield an ingenol-rich fraction (Upadhyay et al., 1977), which was evaporated to dryness.

The 0- and 6-hr fermentation samples from bovine digesta not treated with antibiotic were analyzed. Because there were no significant differences between the 6- and 12-hr fermentation samples in the toxicity assays, we did not analyze the 12-hr sample. Each fermentation sample was hydrolyzed to yield an ingenol-rich fraction (0.8 and 0.9 g for 0- and 6-hr fermentation samples, respectively). The 0- and 6-hr fermentation samples from the antibiotic-treated rumen digesta were not hydrolyzed, so that we were able to detect potential differences with respect to types of ingenols in the fermentation samples.

Thin-Layer Chromatography/Solid Phase Extraction. For the nonfermented LS aqueous extract, normal-phase preparative thin-layer chromatography (TLC;

TABLE 1. OUTLINE OF SOLVENT SYSTEM FOR SOLID PHASE EXTRACTION

Fraction	% MeOH	% H ₂ O	% ACN
1	100	0	0
2	0	100	0
3 ^a	0	0	100
4	0	100	0
5 ^b	0	50	50
6	0	25	75

^a Leafy spurge sample (2.0 mg 1 ml⁻¹ ACN) was added at this time.

^b High-performance liquid chromatographic analysis confirmed the presence of ingenol in fraction 5. ACN = acetonitrile.

20 × 20 cm plate; 1000 pm) was used to detect ingenol by comparison with an ingenol standard. Sample was applied as a continuous band on preparative TLC plates; ingenol standard was applied as a reference. For this experiment, the ingenol-rich fraction from the CH₂Cl₂ extract (total extract for seven plates was 662 mg) was applied on seven preparative TLC plates. The plates were developed in a chamber containing hexane and isopropyl alcohol (2:1, v/v). They were analyzed and compared to the ingenol standard. The band corresponding to the ingenol standard was scraped off the plate, ground with mortar and pestle, extracted three times with CH₂Cl₂ and once with methanol, then filtered through Whatman 41 paper to remove silica material. This dried extract (2.0 mg) was dissolved in acetonitrile (1 ml) and processed through reversed-phase solid phase extraction (RP-SPE) to remove color and interfering material from samples prior to high-performance liquid chromatography (HPLC). A series of solvents was processed through SPE (Table 1). Each eluate from SPE was evaporated, redissolved in acetonitrile, and analyzed for ingenol by HPLC. Nonhydrolyzed samples were processed in the same manner via SPE. Hydrolyzed fermentation samples were not processed through SPE, but rather a guard column was used during the HPLC process to remove interfering materials.

High-Performance Liquid Chromatography. Each fraction eluted from SPE was analyzed (20 µl per injection) using a reversed-phase HPLC column (Econosil C18; 250 mm × 4.6 mm; 5 µm). The nonfermented aqueous extract of LS was used to optimize HPLC separation of ingenol, yielding the following chromatographic program with gradient elution: solvent A (acetonitrile) at 100% for 5 min, then decreased gradually to 60% in 45 min; solvent B (water) was increased from 0 to 40% in the 45-min gradient elution. Column effluent was monitored at 220–240 nm by using a photodiode array detector. Ingenol (Sigma, 1 mg in 1 ml of acetonitrile) was used as the standard. The nonhydrolyzed samples (0.5 mg each) were redissolved in 100 µl acetonitrile, and 15 µl were injected into the HPLC.

Standard Curve of Ingenol Concentration. A standard curve was generated using the solvent system described by Vogg et al. (1999) to determine if the concentration of ingenol esters was increased or decreased during exposure to rumen microbes. Four ingenol standard concentrations (0.0005–0.0375 mg ml⁻¹) were analyzed and used to construct a standard curve.

Determination of Ingenol Concentration in Samples. Ingenol in fermentation samples was determined by a known addition method. Five microliters of ingenol standard (1 mg ml⁻¹ acetonitrile) were dissolved in 95 µl of acetonitrile; Fifteen µl of this were injected into the HPLC to determine the peak area of ingenol. Next, 5 µl of ingenol standard (1 mg ml⁻¹) and 95 µl of each hydrolyzed sample (3 mg dissolved in 1 ml acetonitrile) were combined. Fifteen microliters of each combined sample (equivalent to 0.04275 8(di(m)(di)be5.7(e)(te.5(i)0n2.4(e)0(-429

The instrument shook the plates at maximum agitation for 30 sec prior to each reading of culture turbidity (A_{540}) at intervals of 15–30 min over a period of 16–18 hr, depending on the individual experiment. Methods for calculation of growth rates and for statistical comparisons have been described previously (Weimer and Abrams, 2001).

RESULTS AND DISCUSSION

Toxicity of Leafy Spurge after Fermentation with Normal or Modified Bovine Ruminal Digesta. In assay 1, LS was more toxic than alfalfa ($P < 0.001$; Figure 1), and its toxicity was increased by exposure to bovine ruminal microorganisms for 6 hr ($P < 0.001$) or 12 hr ($P = 0.008$). Observed toxicity was similar ($P = 0.20$) in 6- and 12-hr fermentations. These results indicate that microbial metabolism of chemicals in LS made them more toxic.

In the second assay, adding neomycin sulfate to the mixture did not affect the outcome ($P = 0.65$; Figure 2). LS toxicity increased following 6 hr of exposure to either untreated ($P < 0.001$) or antibiotic-treated bovine ruminal digesta ($P = 0.02$). The addition of neomycin sulfate to the mixtures was intended

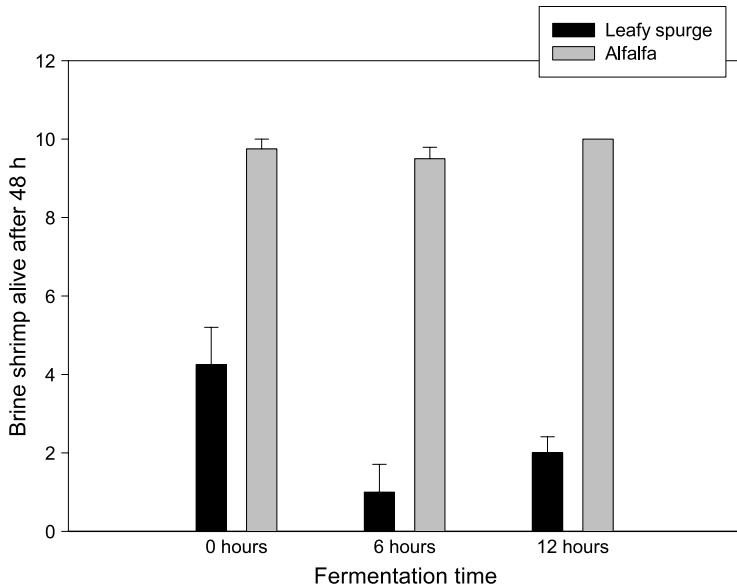


FIG. 1. Number of live brine shrimp in response to extracts of leafy spurge or alfalfa fermented with modified cattle rumen digesta for 0, 6, or 12 hr (assay 1). Bars represent the SE of the means.

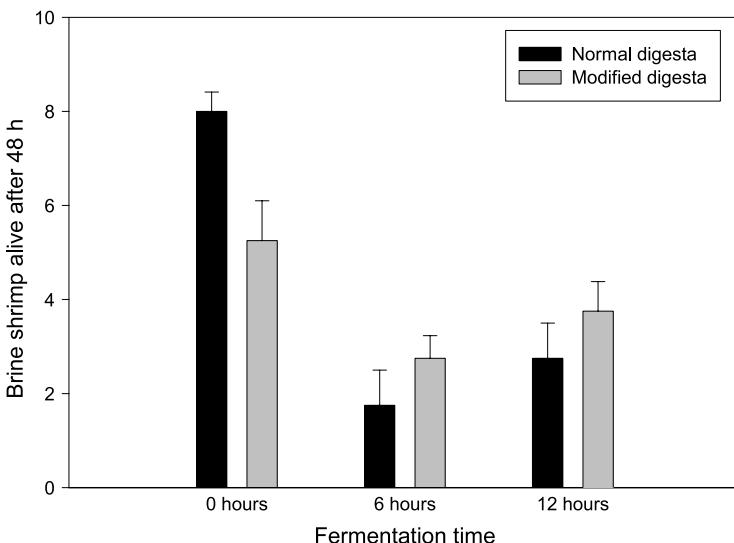


FIG. 2. Number of live brine shrimp in response to extracts of leafy spurge fermented with normal or modified cattle rumen digesta for 0, 6, or 12 hr (assay 2). Bars represent the SE of the means.

to suppress gram-negative bacteria. Assuming that gram-negative bacteria in the digesta were suppressed, this appeared to have no effect on rumen microbial interaction with toxins in leafy spurge. Neomycin blocks protein synthesis in sensitive bacteria, but over the short incubation times used, the bacteria present in the digesta may have already synthesized the proteins they used to metabolize or modify compounds in LS.

Compounds in LS appeared to become more toxic as time of exposure to ruminal microbes increased from 0 to 6 hr. Results from the second assay were consistent with the first in that microbial metabolism of LS compounds appeared to make them more toxic.

This increased toxicity may be caused by ruminal microbes converting compounds in LS into more toxic ones. The ability of ruminal microbes to detoxify or increase the toxicity of plant compounds is well known (James et al., 1975; Duncan and Milne, 1992; Smith, 1992; Wachenheim et al., 1992).

Ingenol Content in Leafy Spurge Following Fermentation with Bovine Rumen Digesta. Retention times from the ingenol standard and the non-fermented aqueous extract of LS were compared, and it was determined that Fraction 5 eluted from SPE contained parent ingenol. The standard curve developed with a HPLC Solvent System described by Vogg et al. (1999) generated a linear detector response with concentration that was used to quantify

ingenol in the hydrolyzed 0- and 6-hr fermentation samples. Results from the sample enrichment technique indicated that the 0-hr fermentation sample contained an average of 0.0024 mg of ingenol/mg sample injected (Figure 3); the 6-hr hydrolyzed fermentation sample contained 0.0011 mg of ingenol/mg sample injected (Figure 4). These values correlated to 0.0074 and 0.0038% ingenols in the original LS and cattle digesta mixture for the 0- and 6-hr fermentation samples, respectively, and they fall within the range of ingenol concentrations found in LS by others. Seip and Hecker (1982) found 0.0005% ingenols in LS, and Upadhyay et al. (1977) found 1.0% ingenols in leafy spurge. The lower concentration of ingenols in the 6-hr fermentation sample could be a result of several factors. Ruminal microbes may have converted ingenols into other toxic compounds, as evidenced by results from the toxicity assays. In each sample, there were peaks that eluted after ingenol, which indicates that these

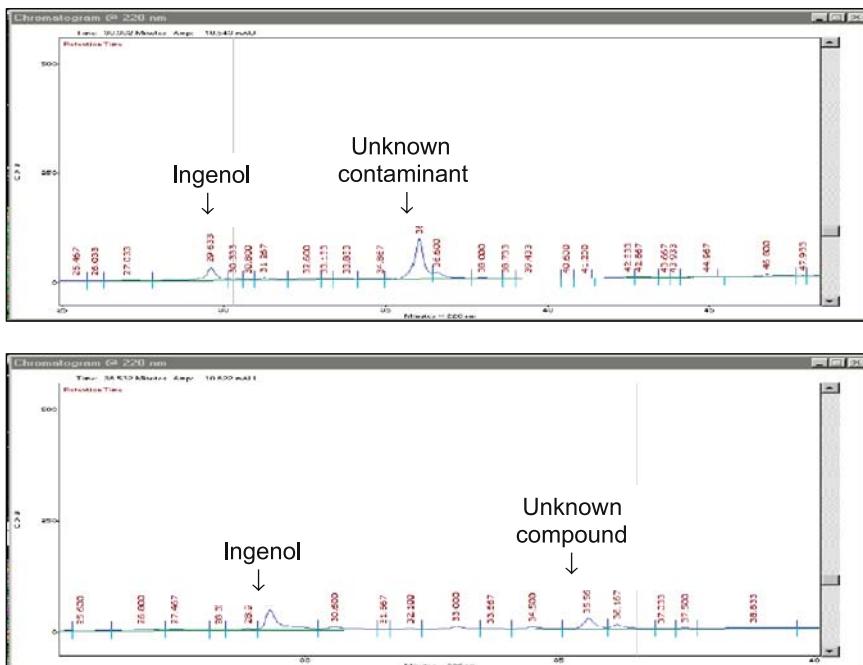


FIG. 3. High-performance liquid chromatogram (HPLC) of ingenol standard: solvent system II; 5 μ l dissolved in 95 μ l of acetonitrile; 15 μ l injected. Average peak area of ingenol = 696,783 AUP; retention time = 29.633 min (top). HPLC chromatogram of ingenol standard (5 μ l) + 0 hr hydrolyzed fermentation sample (95 μ l); 15 μ l injected; solvent system II. Average peak area of ingenol = 948,438 AUP; retention time = 29.300 min (bottom figure).

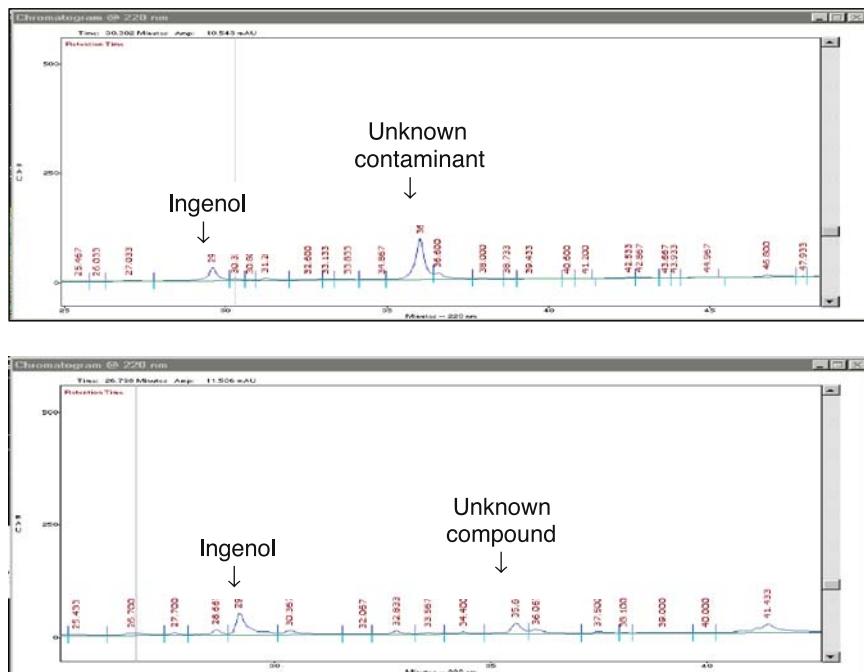


FIG. 4. HPLC chromatogram of ingenol standard: solvent system II; 5 μ l dissolved in 95 μ l of acetonitrile; 15 μ l injected. Average peak area of ingenol = 696,783 AUP; retention time = 29.633 min (top). HPLC chromatogram of ingenol standard (5 μ l) + 6 hr hydrolyzed fermentation sample (95 μ l); 15 μ l injected; solvent system II. Average peak area of ingenol = 893,311 AUP; retention time = 29.200 min (bottom figure).

unknown compounds are less polar than ingenol. Rumen microbes may have transformed ingenol into less polar derivatives. Less polar compounds cross cell membranes easier, and this may explain the higher toxicity of extracts of the fermented mixtures. Alternatively, there may be other toxic compounds in leafy spurge that occur at low levels and are less polar than ingenols.

For the fermentation samples that were not hydrolyzed, ingenol esters present in both the 0- and 6-hr samples showed similar retention times (Figure 5). In each of these samples (from SPE Fraction 5), there were two significant esters present. These esters can passively diffuse through hydrophobic mammalian membranes and enter cells where their toxicity would alter cellular activities (Campbell, 1999). Hasler et al. (1992) demonstrated that ingenol esters activated protein kinase C and were biologically active in cell development, cell-to-cell communication, epidermal growth-factor binding, arachidonic acid metabolite release, and ornithine decarboxylase activity.

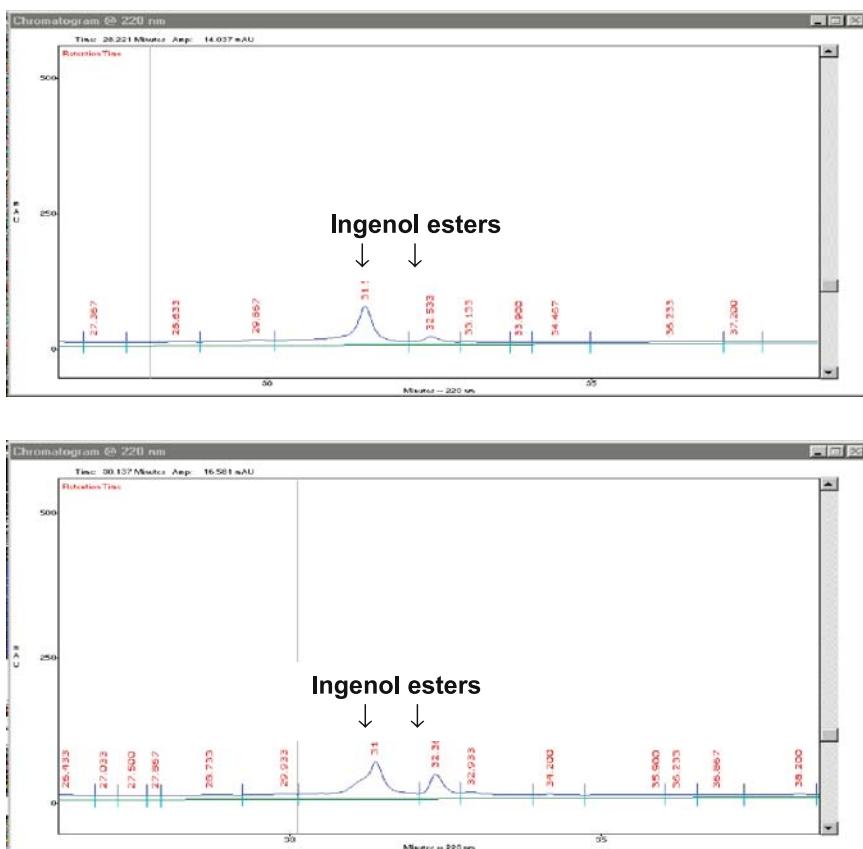


FIG. 5. HPLC chromatogram of 0-hr leafy spurge fermentation sample, nonhydrolyzed (from SPE fraction 5). Five milligrams dissolved in 100 μ l acetonitrile; 15 μ l injected; solvent system II. Ingenol ester 1 retention time = 31.533 min; ester 2 retention time = 32.533 min (top). HPLC chromatogram of 6-hr leafy spurge fermentation sample, nonhydrolyzed (from SPE fraction 5). Five milligrams dissolved in 100 μ l acetonitrile; 15 μ l injected; solvent system II. Ingenol ester 1 retention time = 31.400 min; ester 2 retention time = 32.367 min (bottom figure).

Effect of Leafy Spurge Extract on Growth of Pure Cultures of Rumenal Bacteria. The leafy spurge extract had a small inhibitory effect ($P < 0.05$) on the growth rate of only one of the six rumen bacterial strains tested (*Selenomonas ruminantium*; Table 2). The extract also altered ($P < 0.05$) maximum culture turbidity for this strain as well as for *Streptococcus bovis*, but not for the other four strains of rumen bacteria. It appears that LS does not contain compounds

TABLE 2. EFFECT OF PURIFIED LEAFY SPURGE EXTRACT (LSE; 0.01 mg/ml) ON THE GROWTH OF PURE CULTURES OF RUMINAL BACTERIA

Bacterial strain	Substrate	μ_{\max} (h ⁻¹)			Maximum OD ₆₀₀		
		-LSE	+LSE	P	+LSE	-LSE	P
<i>Butyrivibrio fibrisolvens</i> H17c	Glucose	0.259	0.284	NS	0.932	0.906	NS
<i>Fibrobacter succinogenes</i> S85	Cellobiose	0.230	0.231	NS	1.375	1.374	NS
<i>Lachnospira multipara</i> 40	Glucose	0.655	0.636	NS	1.871	1.923	NS
<i>Prevotella ruminicola</i> B14	Glucose	0.587	0.579	NS	1.390	1.370	NS
<i>Selenomonas ruminantium</i> D	Glucose	0.609	0.588	<0.05	0.587	0.658	<0.05
<i>Streptococcus bovis</i> JB-1	Glucose	1.268	1.231	NS	1.113	1.042	<0.05

Results are from eight replicate cultures of each strain grown in microtiter plates.

ns = not significant at P = 0.05.

that inhibit common species of bacteria known to be responsible for a wide array of fermentative activities in the rumen. Therefore, it appears unlikely that aversion-inducing compounds in LS are seriously inhibiting normal forage digestion in the rumen of cattle that could, thus, cause them to learn to avoid consuming additional LS. These results are consistent with those of Thomas et al. (1994) who concluded that leafy spurge had no negative effects on rumen digestion in sheep when it made up 50% of their diet.

In summary, fermentation of LS with bovine ruminal digesta generally increased the toxicity of extracted compounds to brine shrimp, a general model system for studies of animal toxicity. This indicates that interactions between LS toxins and rumen microbes are may be increasing the toxicity of these compounds to cattle. Introduction of a gram-negative antibiotic into the LS and cattle rumen digesta mixture did not increase or decrease toxicities of this mixture regardless of fermentation length. Levels of toxic ingenol that we observed in LS before or after exposure to bovine ruminal digesta were consistent with levels of ingenols others have reported for LS. Finally, an aversion-inducing extract of LS had little negative effect on several common species of rumen bacteria comprising a broad array of ruminal metabolic activities. This indicates that the mechanism of action of aversion-inducing compounds in LS is not inhibition of forage digestion by rumen microbes but rather is direct toxicity to cattle.

Acknowledgments—We thank Kristine Nichols, Douglas Ranynie, Igor Sergeev, and anonymous reviewers for their helpful comments on the manuscript and Kjersten Grong for help with the brine shrimp toxicity assays.

REFERENCES

- ANDERSON, J. E., GOETZ, C. M., and MC LAUGHLIN, J. L. 1991. A blind comparison of simple bench-top bioassays and human tumour cell cytotoxicities as antitumor prescreens. *Phytochem. Anal.* 2:107–111.
- CAMPBELL, M. K. 1999. Biochemistry. Saunders College Publishing, Philadelphia.
- CARLSON, J. R. and BREEZE, R. G. 1984. Ruminal metabolism of plant toxins with emphasis on indolic compounds. *J. Anim. Sci.* 58:1040–1049.
- CRAIG, A. M., LATHAM, C. J., BLYTHE, L. L., SCHMOTZER, W. B., and O'CONNOR, O. 1992. Metabolism of toxic pyrrolizidine alkaloids from tansy ragwort in bovine ruminal fluid under anaerobic conditions. *Appl. Environ. Microbiol.* 58:2730–2736.
- DUNCAN, A. J. and MILNE, J. A. 1992. Rumen microbial degradation of allyl cyanide as a possible explanation for the tolerance of sheep to brassica-derived glucosinolates. *J. Sci. Food Agric.* 58:15–19.
- HALAWEISH, F. T., KRONBERG, S. L., HUBERT, M. B., and RICE, J. A. 2002. Toxic and aversive diterpenes of *Euphorbia esula*. *J. Chem. Ecol.* 28:1599–1611.
- HASLER, C. M., GEZA, A., and BLUMBERG, P. M. 1992. Specific binding to protein kinase C by ingenol and its induction of biological responses. *Cancer Res.* 52:202–208.
- HEIN, D. G. and MILLER, S. D. 1992. Influence of leafy spurge on forage utilization by cattle. *J. Range Manag.* 45:405–407.
- JAMES, L. F., ALLISON, M. J., and LITTLEDIKE, E. T. 1975. Production and modification of toxic substances in the rumen, pp. 576–590, in I. W. McDonald and A. C. I. Warner (eds.). Digestion and Metabolism in the Ruminant. University of New England Publishing Unit, Armidale, New South Wales, Australia.
- KIRBY, D. R., HANSON, T. P., and HULL-SIEG, C. 1997. Diets of Angora goats grazing leafy spurge *Euphorbia esula*-infested rangeland. *Weed Technol.* 11:734–738.
- KRONBERG S. L. 1999. Rumen microbial metabolism affects diet selection of ruminants, pp. 460–461, in D. Eldridge and D. Freudenberger (eds.). Proceedings of the 6th International Rangeland Congress. Townsville, Australia.
- KRONBERG, S. L. and WALKER, J. W. 1993. Ruminal metabolism of leafy spurge in sheep and goats: a potential explanation for differential foraging on spurge by sheep, goats, and cattle. *J. Chem. Ecol.* 19:2007–2017.
- KRONBERG, S. L., MUNTFERING, R. B., AYERS, E. L., and MARLOW, C. B. 1993a. Cattle avoidance of leafy spurge: a case of conditioned aversion. *J. Range Manag.* 46:364–366.
- KRONBERG, S. L., MUNTFERING, R. B., and AYERS, E. L. 1993a. Feed aversion learning in cattle with delayed negative consequences. *J. Anim. Sci.* 71:1767–1770.
- LANDGRAF, B. K., FAY, P. K., and HAVSTAD, K. M. 1984. Utilization of leafy spurge (*Euphorbia esula*) by sheep. *Weed Sci.* 32:348–352.
- LYM, R. G. and KIRBY, D. R. 1987. Cattle foraging behavior in leafy spurge infested rangeland. *Weed Tech.* 1:314–318.
- MC LAUGHLIN, J. L., CHANG, C., and SMITH, D. 1991. "Bench-Top[®] bioassays for the discovery of bioactive natural products: an update. *Stud. Nat. Prod. Chem.* 9:383–405.
- MEYER, B. N., FERRIGNI, N. R., PUTNAM, J. E., JACOBSEN, L. B., NICHOLS, D. E., and MC LAUGHLIN, J. L. 1982. Brine shrimp: a convenient general bioassay for active plant constituents. *J. Med. Plant Res.* 45:31–34.
- PASS, M. A., MAJAK, W., MUIR, A. D., and YOST, G. S. 1984. Conversion of 3-nitropropanol to 3-nitropionic acid in cattle and sheep. *Toxicol. Lett.* 23:1–7.

- RUSSELL, J. B. and STROBEL, H. L. 1988. Effects of additives on *in vitro* ruminal fermentations: a comparison of monensin and bacitracin, another gram-positive antibiotic. *J. Anim. Sci.* 66:552–558.
- SAS. 1988. SAS STAT User's Guide. Statistical Analysis System Institute, Cary, NC.
- SEIP, E. H. and HECKER, E. 1982. Skin irritant ingenol esters from *Euphorbia esula*. *J. Med. Plant Res.* 46:215–218.
- SMITH, G. S. 1992. Toxicification and detoxification of plant compounds by ruminants: an overview. *J. Range Manag.* 45:25–30.
- THOMAS, V. M., CLARK, C. K., KNOTT, R. W., and OLSON, B. 1994. Influence of leafy spurge on ruminal digestion and metabolism and blood metabolite profiles in sheep. *Sheep Goat Res. J.* 10:168–172.
- TILLEY, J. M. A. and TERRY, R. A. 1963. A two stage technique for the *in vitro* digestion of forage crops. *J. Br. Grassl. Soc.* 18:104–111.
- UPADHYAY, R. R., KHALESI, K., KHARAZI, G., and GHAI SARZADEH, M. 1977. Isolation of ingenol from the plants of *Euphorbiaceae*. *Indian J. Chem.* 15:294–111, 1977.
- VOGG, G., MATTES, E., ROTHENBURGER, J., HERTKORN, N., ACHATZ, S., and SANDERMAN, H., JR. 1999. Tumor promoting diterpenes from *Euphorbia leuconeura*. *Phytochemistry* 51:289–295.
- WACHENHEIM, D. E., BLYTHE, L. L., and CRAIG, A. M. 1992. Effects of antibacterial agents on *in vitro* ovine ruminal biotransformation of the hepatotoxic pyrrolizidine alkaloid jacobine. *Appl. Environ. Microbiol.* 58:2559–2564.
- WALKER, J. W., KRONBERG, S. L., AL-ROWAILY, S. L., and WEST, N. E. 1994. Comparison of sheep and goat preferences for leafy spurge. *J. Range Manag.* 47:434–439.
- WEIMER, P. J. and ABRAMS, S. M. 2001. *In vitro* fermentation of polydextrose by bovine ruminal microorganisms. *Anim. Feed Sci. Technol.* 93:115–123.